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Analysis for Plasma Protein Biomarkers Following an Accidental Human Exposure to Sulfur Mustard

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Abstract

Following an accidental human exposure to a vesicating agent, plasma samples were analyzed for specific biomarkers of sulfur mustard. One individual suffered chemical burns over 6.5% of the body surface area and required hospitalization; the second individual developed a single, small blister. Plasma specimens from both individuals were examined using two different assays. The first assay targeted sulfur mustard adducts to cysteine-34 of albumin using affinity chromatography, enzyme digestion, and analysis of the alkylated peptide fragment using liquid chromatography–tandem mass spectrometry. The second assay targeted alkylation sites of glutamic and aspartic acids of plasma proteins. Following precipitation of plasma proteins, the sulfur mustard adducts were cleaved from the protein using base, derivatized, and analyzed using gas chromatography–mass spectrometry. Samples obtained over a 42-day period from the individual requiring hospitalization produced positive results for sulfur mustard adducts using both assays. Observed levels of the sulfur mustard biomarker decreased by approximately 75% between days 2 and 42 for both assays. Samples obtained over a six-day period from the individual with a single, small blister produced positive results for the albumin adduct assay. Observed levels were much lower than levels from the hospitalized patient. Blood samples from suspected human exposures to sulfur mustard have only rarely been made available for analysis by sensitive and specific laboratory assays. The data presented here add significantly to the small database of information that currently exists on human biomarkers of sulfur mustard exposure, linking a well-documented exposure event with levels of plasma protein adducts.

Introduction

Human exposures to the chemical warfare agent sulfur mustard (HD) have generally been associated with military use of the vesicating agent in World War I (WWI). More recently, military use of HD was documented in the Iran–Iraq conflicts of the 1980s and against the Kurdish civilian population in Iraq. In addition, sulfur mustard poses a potential public health threat from terrorist activities or from accidental contact. A number of published reports have chronicled accidental human exposures to HD that have occurred from a surprising variety of situations, including exposures to fishermen in the Baltic Sea (1), accidental contact with WWI munitions (2,3), a chemical research laboratory accident (4), and worker exposure at a chemical weapon storage facility (5). Despite the number of military or accidental human encounters with HD over the years, only rarely has blood been made available for analysis of potential biomarkers of HD exposure. Prior to the incident described in this report, all but a single blood specimen examined for HD biomarkers had been obtained from archived samples taken from Iranian victims of suspected HD exposure. Blood components from some of the Iranian victims were frozen at the time of collection. Years later as new analytical methods for HD verification were developed using in vitro or animal models, research scientists were able to access the preserved blood specimens to validate the presence of the HD biomarkers in samples from exposed humans.

An important chemical reaction of sulfur mustard is the formation of a highly reactive sulfonium ion that is produced following cyclization of an ethylene group. The sulfonium ion can in turn react with nucleophiles, such as water, or combine with nucleophilic sites of macromolecules to produce hydroxyethylthioethyl (HETE) esters (6). The resulting free metabolites and alkylated macromolecules can be exploited for analysis in biomedical samples such as blood, urine, or tissue (7–9). Whereas urinary metabolites undergo relatively rapid elimination from the body, blood components offer macromolecular biomarkers that have the potential to be used for verification of sulfur mustard exposure long after the exposure incident. Three different approaches have been utilized for blood macromolecular analysis. The entire alkylated macromolecule such as protein or DNA with the HD adduct(s) intact

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can be analyzed. Alternatively, the macromolecule can be subjected to enzymatic or chemical digestion to produce a smaller fragment that still retains the adduct. A third approach has been to cleave the adduct from the macromolecule and analyze the unbound adduct in a manner similar to that used for free metabolites. Several of these approaches have been used to identify and measure HD biomarkers from blood samples obtained from victims of HD exposure.

Various hemoglobin-adduct biomarkers for HD exposure have been extensively studied (10–12), in many cases using mass spectrometry (MS) (3,11–15). The (HETE)histidine adducts were found in archived blood samples collected from four hospitalized Iranian casualties 5–10 days after exposure and from one individual 2 days after an accidental exposure to a WWI munition (3). The (HETE)valine adduct of hemoglobin was also observed in the same five individuals (3). Archived blood samples that were obtained from two hospitalized Iranian casualties 22 and 26 days following a suspected HD exposure tested positive for both the (HETE)valine adduct and for HD DNA adducts (16). The most recent report on the analysis of archived blood samples obtained from Iranian casualties also indicated positive results for the (HETE)valine adducts. The samples were obtained from nine individuals and were collected 8–9 days after the suspected HD exposure (17).

Human serum albumin (HSA) was found to be alkylated by sulfur mustard at the cysteine-34 position. Following enzymatic digestion of HSA, one of the resulting peptide fragments is a tripeptide of the sequence Cys-Pro-Phe that contains the HETE adduct. The tripeptide can be analyzed using liquid chromatography–electrospray-tandem MS (LC–ESI-MS–MS) (18,19) and was one of the two assays used in this report to examine plasma samples from a suspected human exposure to a vesicating agent. The only previous use of the albumin adduct assay for verification of human exposure to HD was the analysis of archived Iranian blood samples from nine individuals. Blood samples tested positive for HD-alkylated albumin (18) and also tested positive for the (HETE)valine adduct to hemoglobin (17).

It has previously been shown that sulfur mustard adducts of glutamic and aspartic acids to keratin could be cleaved from the protein using base (20). Using a similar approach, precipitated proteins from plasma can be treated with base to liberate the HETE adduct from the protein. Upon release, the cleaved adduct forms thiodiglycol and following derivatization can be analyzed using gas chromatography–mass spectrometry (GC–MS) (21). Regardless of the specific protein source(s) of liberated HETE esters, once the adducts are unbound from the protein they can be analyzed in a manner similar to those used for examining free metabolite concentrations. Any free TDG that might have been present in the initial blood sample would be lost during the initial protein precipitation and washing steps of the sample preparation. The removal of free metabolites prior to the cleavage of the adduct from the protein is significant because background levels of TDG have been reported in blood from unexposed individuals (22). This was the second assay used to examine blood samples for HD biomarkers in this report. It was the first use of this recently developed assay for the verification of a human HD exposure.

The data presented in this report resulted from the analysis of blood samples obtained from two ordnance technicians involved in the destruction of a WWI munition in July 2004. A liquid leaking from the munition came into dermal contact with the two technicians. In less than 24 h, clinical evidence clearly indicated that the liquid was a vesicating agent. Patient 1 suffered chemical burns over approximately 6.5% of the body and required hospitalization, while Patient 2 had only a single, small blister and was not hospitalized. Blood samples from the patients were found to be positive for sulfur mustard biomarkers using the albumin method (Patients 1 and 2) and plasma protein adduct method (Patient 1), and in the case of Patient 1, blood samples remained positive for 42 days following the incident. This well-documented exposure event provided a rare opportunity to compare the two protein adduct methods and to confirm the retrospectivity of these methods. This report complements concurrent studies of urinary metabolites of HD from these individuals (23).

Experimental

Patient samples

The results presented in this report were generated from the analysis of blood samples obtained from two explosive ordnance technicians who were part of a team tasked with the destruction of a suspected WWI 75-mm munition. The munition had been discovered in a clamshell driveway and was believed to have originated from material dredged from a seafloor dumping area. Following demolition procedures, the two individuals came into contact with a brown oily liquid that was found leaking from the remnants of the munition. During the disposal operation, there were no complaints of eye and/or throat irritation or breathing difficulties by any of the ordnance team members. The clinical sequence of events for the individual with the more severe injuries has previously been reported (24–26), but will be described in brief here. Within 2 h of the munition's destruction, one of the individuals (a 35-year-old male) noticed a tingling sensation on one arm and then showered. The next morning (approximately 14 h after the liquid contact), he had developed painful areas of the hand with noticeable reddening and the formation of small blisters. The blisters continued to grow and coalesce, and the patient was subsequently transferred to a regional burn center. The erythema and blistering area on the patient was estimated to be 6.5% body surface area. The patient never suffered any ocular or respiratory complications; consequently, it appeared that the patient's injuries were only the result of a cutaneous liquid exposure. The second individual had a small, single blister and was not hospitalized.

The collection and analysis of blood specimens from the exposed individuals in this report was initiated following the informed consent of both patients. Blood samples were collected from Patient 1 on days 2–10, 29, 35, and 42 after the exposure incident. Blood samples for Patient 2 were collected on days 2, 4, and 7 after exposure. The samples were collected in purple top vacutainers containing K_3EDTA. Upon receipt, each blood

specimen was separated into red blood cell (RBC) and plasma fractions by centrifugation for 10 min at 10°C and stored at −70°C. Blood samples received from days 29, 35, and 42 appeared to be hemolyzed and were spun under the same conditions for an additional 20 min. The day 42 sample separated into plasma and RBC fractions, but samples from days 29 and 35 did not separate into distinct layers. An upper 1-mL portion of the hemolyzed sample was removed from day 29 and 35 samples for analysis. Coincidentally, the chemical exposure incident occurred in the very same month that the methods described below were published. Where sample volumes were sufficient, aliquots of the blood were sent to both the U.S. Army and CDC laboratories to be examined for HD biomarkers using the plasma protein adduct or albumin adduct method, respectively.

Analysis of plasma samples for sulfur mustard adducts to HSA

The analysis of plasma samples for HD adducts to albumin followed the method of Noort et al. (18), and incorporated the modifications to the original method as described by Noort et al. (19). Sample preparation and analysis was performed as described previously (19) except where noted. In brief, HSA was isolated from plasma samples (0.5 mL) using affinity chromatography on 1 mL HiTrap™ Blue columns (GE Healthcare, Piscataway, NJ), then desalted on PD-10 (Sephadex G25) columns, and digested using Pronase (protease from *Streptomyces griseus*, Sigma-Aldrich, St. Louis, MO) at an enzyme/substrate ratio of 1:3 in 50mM ammonium bicarbonate at pH 8.5 for 2 h at 37°C. New HiTrap Blue and PD-10 columns were used for each sample, to avoid carryover issues. A tripeptide fragment, Cys-Pro-Phe, containing the HD alkylation site cysteine-34 was subsequently analyzed using LC–ESI-MS–MS. Fragmentation of the protonated molecular ion, m/z 470, to yield the HETE fragment ion at m/z 105 was monitored using the multiple reaction monitoring (MRM) mode on a 4000QTrap MS (Applied Biosystems, Foster City, CA). The LC system and MS parameters were as described previously for a similar API4000 MS system (19). Standards for this method were generated by spiking whole blood with HD dissolved in acetonitrile, followed by incubation at 37°C for 2 h (18). Internal standard materials using fully deuterated HD were prepared in the same manner. MRM transitions of (HETE-d_8)-Cys-Pro-Phe, m/z 478 to HETE-d_8, m/z 113 were monitored for isotope-dilution quantification. The lower limits of detection and quantitation for the assay were 1.5nM-ExB and 4.5nM-ExB, respectively, using the (ExB) to indicate that these units are based on standards obtained from in vitro exposures of human whole blood with HD using the described protocol (18). Standard curves were prepared by dilution of Pronase-digested 1000nM-ExB plasma preparations with appropriate volumes of digested CDC blank plasma preparations, such that patient samples were bracketed. For example, the lowest level patient samples were quantitated using a standard curve having six evenly spaced levels between 5 and 30nM-ExB, whereas the highest level patient samples were quantitated using nine levels covering the range of 100–1000nM-ExB. The d_8 internal standard was spiked at 200nM-ExB in all cases.

Human blood used for control samples and the preparation of standards was obtained from a blood bank and was determined to have zero detectable levels of any HD adducts. A small reference range study of unexposed individuals indicated no detectable levels of the alkylated tripeptide or background interferences in serum samples obtained from 80 individuals (19).

A single exponential decay curve was fitted to all measured values for Patient 1 for the (HETE)tripeptide over the sample collection period using Microsoft Excel, based on the assumption that each sample was collected at the same time of day. The half-life of the albumin adduct was estimated from this decay curve.

Analysis of plasma samples for sulfur mustard adducts to plasma proteins

Plasma samples were prepared and analyzed for HD protein adducts exactly as described using the method of Capacio et al. (21), except for the addition of a final concentration step as noted. Pooled human plasma used for controls and the preparation of standards was obtained from Innovative Research (Southfield, MI). In brief, plasma was washed with acetone to precipitate proteins. After allowing the pellet to dry, the plasma proteins were treated with base to liberate the sulfur mustard HETE adduct from the protein as thiodiglycol (TDG), followed by addition of TDG-d_8 as an internal standard. TDG and TDG-d_8 were subsequently derivatized using pentafluorobenzoyl chloride and analyzed using negative-ion chemical ionization GC–MS. The derivatized adduct and TDG-d_8 internal standard were measured as molecular anions using selected ion monitoring (SIM) at m/z 510 and m/z 518, respectively. Standards for this method were prepared by spiking plasma with HD dissolved in saline, followed by incubation at 37°C for 18 h. The reported lower limit of quantitation for the isotope-dilution plasma assay was 25nM-ExP, using the (ExP) to indicate that these units are based on standards obtained from in vitro exposures of HD in human plasma using this protocol (21). By concentrating the final sample solution under nitrogen, the lower limit of quantitation was lowered to 20nM-ExP.

Graphic representations, linear regression analyses, and non-linear curve fitting analyses were carried out with GraphPad Prism software (version 3.02, GraphPad Software, San Diego, CA). Concentration-time data were fit to a one- or two-phase exponential decay equation. Selection of the best-fit equation was accomplished by comparison with an F test. Statistical significance was considered at a level of $P < 0.05$. Calculations for TDG concentrations and other descriptive statistics were accomplished with Microsoft Excel 2000.

Results and Discussion

The exposure incident reported here involved two individuals that were accidentally exposed to a WWI munition in 2004, with clinical symptoms that have been very well documented (24–26). Several factors contributed to a situation

whereby this particular incident differed in several critical aspects from previously reported incidents: 1. biomarker levels were measured from two individuals from the same exposure incident, but with two very different levels of injuries; 2. both urine and blood samples were obtained for laboratory testing; and 3. multiple timepoints of sample collections were made, including 42 days after HD exposure. The results presented here are only the second reported use of the albumin HD adduct assay for the verification of human exposure to sulfur mustard. Although the application of the plasma protein HD adduct assay had been previously demonstrated by Capacio et al. (21),

using two different animal models, the extrapolation of the assay as a valid human biomarker assay had not been established. This was the first use of the assay for the verification of a human exposure to sulfur mustard.

The adduct-based methods generally have the advantage of retrospectivity, because the adducts are often quite stable and their half-lives may be similar to those of the macromolecules involved. However, the preparation of standard and quality control (QC) materials for quantitative analyses can present some challenges. In vitro exposures of HD can conveniently be made using whole blood or plasma, but the delivery method, the incubation time or the temperature may differ between laboratories. Furthermore, the levels of agent measured following in vivo exposure events may be expressed relative to the way in which the standards and QC materials were made, as opposed to true micromolar concentrations that would be found in vivo.

The HD albumin adduct and plasma protein adduct methods described here used standards prepared from whole blood and plasma incubated at 37°C with HD dissolved in acetonitrile and saline, respectively. To compare these methods, a QC sample was prepared by exposing plasma to 400nM HD using the plasma protein protocol, and was then analyzed using the albumin adduct method. Using suffixes to indicate that the measured concentrations are relative to the standard materials, the 400nM-ExP plasma-based QC sample was measured as 1147 ± 45nM-ExB ($n = 8$) in the albumin adduct method. Thus, the units of measurement appear to differ by a factor of between 2.8-fold and 3-fold for these methods. Although much of this difference can be accounted for by assuming that whole blood is ~50% plasma by volume, it is also likely that other sources of variance exist.

The assay developed by Noort et al. (19) targeted a very specific site of HD alkylation on a specific isolated protein. The target sequence of interest is a tripeptide fragment containing the amino acid cysteine-34 HD alkylation site. Cysteine-34 is the only free thiol group of HSA that is a nucleophilic site and thus capable of reacting with HD (27). Alternatively, the method of Capacio et al. (21) used a more generalized approach regarding both target proteins and HD alkylation sites on those proteins. The method employs a non-specific plasma protein precipitation procedure and then targets HD alkylation of aspartic and glutamic acids of any proteins obtained. Both aspartic and glutamic acid amino acids have free carboxylic acid groups that can react with the electrophilic sulfur mustard to produce HETE esters. HSA is found in great abundance in plasma with reported concentrations between 38 and 53 g/L (28). Additionally, of the 585 amino acids that make up HSA, nearly 17% are either aspartic or glutamic acids (27) although protein conformation would probably restrict or hinder reactions with HD for many of the acids. Consequently, HSA would be expected to be an important source contributing to the total number of HD adducts cleaved using the generalized plasma protein approach.
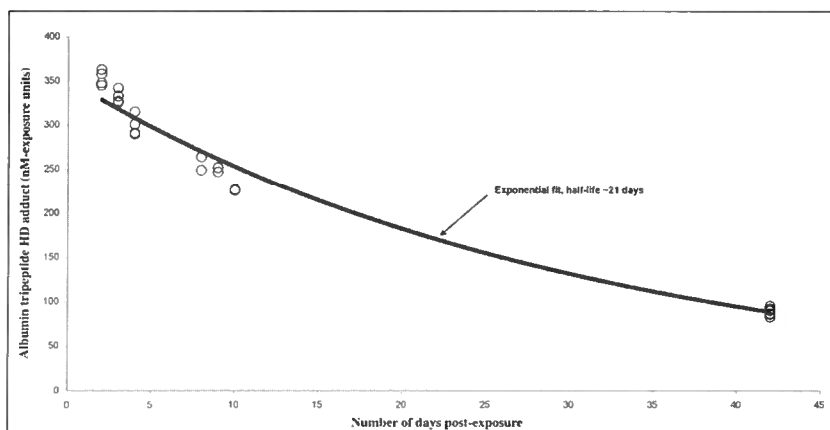
Table I. Observed Concentrations of Sulfur Mustard Biomarkers in Blood for Patient 1

Number of Days After Exposure	HSA Adduct Concentration (nM-ExB)*	Plasma Protein Concentration (nM-ExP)†
2	354 ($n = 4$; SD = 11)	97
3	332 ($n = 4$; SD = 10)	74
4	299 ($n = 4$; SD = 14)	66
5	NS‡	52
6	NS	53
7	NS	61
8	257 ($n = 2$)	52
9	250 ($n = 2$)	46
10	227 ($n = 2$)	38
29§	NS	23
35§	NS	22
42§	90.8 ($n = 8$; SD = 3.0)	20

* Concentration based on in vitro exposure of whole blood to HD, using a specific protocol as described in the text. The measured units 'nM-ExB' are relative to this in vitro protocol. Listed values are the average of multiple analyses by LC–MS–MS of the same prepared sample (n = number of injections; standard deviation (SD) given where $n > 2$).
† Concentration based on in vitro exposure of plasma to HD, using a specific protocol as described in the text. The measured units "nM-ExP" are relative to this in vitro protocol. Listed values are the average of two sample aliquots prepared and analyzed independently.
‡ NS = Not measured due to insufficient sample volume to analyze using both assays.
§ Blood samples were hemolyzed upon receipt. See Experimental section for details.

Figure 1. The concentration-time course (Patient 1) of the alkylated albumin tripeptide fragments. Data points represent repeat LC–ESI-MS–MS analysis of a prepared sample. The curve was fitted assuming a single exponential decay process, using Microsoft Excel.

The albumin adduct assay of Noort et al. (19) was able to detect the HD alkylated tripeptide containing (HETE)Cys-34 in all plasma samples that were analyzed from both individuals. This included a blood specimen that was obtained 42 days after the exposure incident. The highest observed concentration of 354 nM-ExB was found in the first sample received (day 2 after exposure) from Patient 1. The initial blood samples received for both individuals were drawn two days after exposure; several reports containing preliminary results of analysis (26,29,30) mistakenly indicated that initial samples were obtained one day after exposure. Patient 1 concentrations decreased over the next two days to 332 and 299nM-ExB (days 3 and 4, respectively). During the second week after the exposure, observed alkylated tripeptide concentrations continued to decline to 257, 250, and 227nM-ExB on days 8, 9, and 10, respectively. Six weeks after the exposure had occurred (day 42), the observed concentration was 90nM-ExB (Table I). This concentration was still well above the lower limit of quantitation of 4.5nM-ExB of the assay and was a 74% decrease of the concentration found on day 2. The rate of decrease over that time period, estimated as a half-life of 21 days assuming that a single exponential decay was occurring (Figure 1), was consistent with the reported half-life of human albumin of 17–23 days (28) and would indicate that the HD alkylated albumin had the same stability as the native albumin in vivo. Albumin adduct concentrations for Patient 2 over the sample collection period of 2 to 7 days after exposure appeared to remain stable and ranged between 16 and 18nM-ExB (Figure 2).

The HD albumin adduct assay has only been used one time prior to this report on blood obtained from human HD casualties. Blood samples obtained in 1986 from nine Iranian casualties of sulfur mustard exposure were analyzed for the albumin (HETE)cysteine adduct using LC–ESI-MS–MS (18). The same samples were also analyzed for the (HETE)valine adduct of hemoglobin using GC–MS (17). All nine individuals had been hospitalized with skin injuries that were consistent with HD exposure. Several of the casualties also suffered from respiratory difficulties. Blood samples were collected between 8 and 9 days after the exposure incident and then stored at

–70°C. The RBCs had hemolyzed during storage, and albumin isolation was performed without first separating RBCs from plasma. Exposure levels of the patient blood samples were correlated with human whole blood that was exposed to HD in vitro. Adduct levels for both the albumin cysteine adduct and for the hemoglobin valine adduct were in very close agreement with each other. Observed exposure levels were between 0.4–1.8μM and 0.3–2μM (relative exposure units, probably similar to our μM-ExB units) for the albumin and hemoglobin adducts, respectively. By comparison, the highest observed concentration of the albumin (HETE)cysteine adduct for Patient 1 was 0.35μM-ExB on day 2 after exposure. At approximately the same timepoint (8–9 days) as the blood taken from the Iranian casualties, the albumin adduct concentrations in Patient 1 were 0.250–257μM-ExB.

The plasma protein HD adduct assay developed by Capacio et al. (21), was able to detect HD-alkylated plasma proteins in all plasma samples received from Patient 1. Concentrations of the plasma protein adducts based on in vitro exposures of sulfur mustard in human plasma were 97nM-ExP on day 2 and had decreased by 79% (20nM-ExP) on day 42 (Figure 3). The concentration-time profile curve generated from the plasma protein assay indicated a more rapid decrease in HD-alkylated products than the rate observed for the albumin adducts during the first two weeks. By day 8, the concentration of HD adducts had been reduced by 46%, whereas the albumin adduct concentration had only been reduced by 27%. The assay was unable to detect plasma protein adducts in Patient 2 blood samples. Minor modifications were made to the assay to improve the sensitivity since it was anticipated that samples collected several weeks after the exposure might require a greater level of sensitivity than the lower limit of quantitation of 25nM-ExP using the original assay. Blood samples collected from Patient 1 on days 29–42 were indeed found to require the improvement in sensitivity. Concentrations observed over that time period ranged between 20 and 23nM-ExP (Table I). The limited amount of plasma received was insufficient to permit reanalysis of samples for Patient 2 using the modified method. More recently, the lower limit of quantitation of the assay has been improved significantly by Lawrence et al. (31).
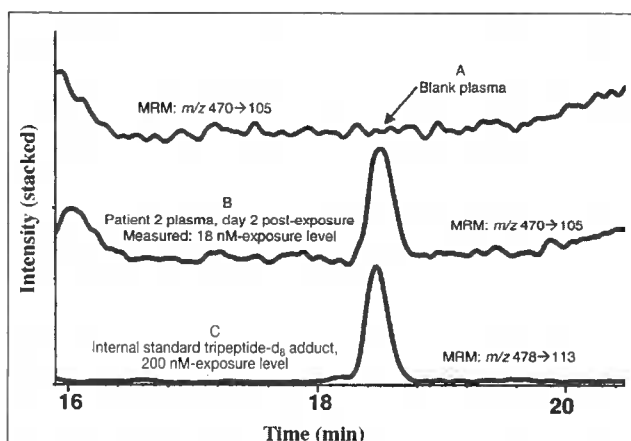

Figure 2. MRM transition ion chromatograms of control blank plasma sample (A), Patient 2, day 2 plasma sample (B), and deuterated internal standard (C). The blank plasma was from the same CDC pool used to prepare the standards.
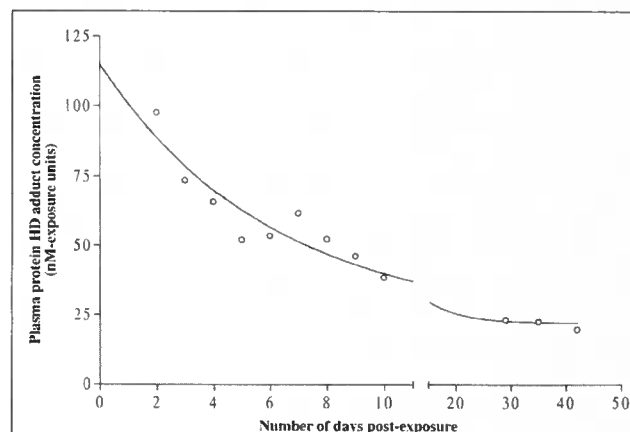

Figure 3. The concentration-time course (Patient 1) of the HETE adducts cleaved from precipitated plasma proteins. The data were fit to a one-phase exponential decay equation using GraphPad Prism software.

The exposure-based concentration values (nM-ExB and nM-ExP units) of the Patient 1 samples reported here differ by a factor of 3.6-fold for the two HD adduct methods on the second day following exposure, while the differences at the later time-points were approximately 5-fold (Table I). The 3.6-fold difference is somewhat greater than the 2.8- to 3.0-fold difference obtained on a QC sample prepared in vitro, but it is not currently known whether this is significant. However, an increasing difference (up to ~5-fold) at the later time-points is consistent with a more rapid loss of the non-specific protein adducts. This may possibly be attributed to shorter half-lives of some of the plasma proteins involved, and/or it might also be the result of various hydrolytic or enzymatic reactions that might release the HETE adduct from aspartic or glutamic acid residues.

The incident provided a valuable opportunity to examine biomarker levels from a single, well-documented chemical exposure event, but one in which two individuals suffered very different levels of injuries. Patient 1 had extensive vesication of the arm and leg. Clinical aspects of the case, including the lack of pain during the chemical exposure, the time sequence of the development of blisters, and the "string of pearls" pattern of the blisters, were strong indicators that the patient had suffered a possible exposure to sulfur mustard. The second patient suffered a single, small blister and in the absence of the first patient's injuries would have offered a much greater challenge to a clinical diagnosis. As expected, the observed HD-alkylated albumin concentrations were much greater for Patient 1. On day 2 after exposure, the observed concentrations were 350 and 18nM-ExB for Patients 1 and 2, respectively. The day 2 concentration for Patient 2 was only 5% of the concentration observed in Patient 1 and was only four times greater than the lower limit of quantitation for the assay. Although the analysis of blood from a low-level exposure to HD offers an analytical challenge, these results indicate that the potential exists for verification of a low-level human exposure to HD as long as a week after the event and possibly longer.

The two individuals in this report are only the second and third casualties of a sulfur mustard exposure to have both urine and blood samples made available for laboratory testing and consequently provided an opportunity for a direct comparison of the sensitivity requirements and longevity of biomarkers in two very different biomedical sample matrices. The urinary metabolite results are detailed in an accompanying article in this volume (23). By day 7, the β-lyase urinary metabolites derived from the reaction of HD with glutathione were still detected in Patient 1, but the observed concentration was approaching the limit of detection for the assay. All urine samples collected from Patient 1 beyond day 11 tested negative for all HD urinary biomarkers. In contrast, both assays used for the analysis of Patient 1 blood samples were able to detect their respective HD biomarkers over the first 10 days at concentrations well above the limit of detection of each method, and both blood assays were able to detect HD biomarkers from the last samples collected at day 42. The β-lyase urinary metabolites were detected in Patient 2 urine through day 7, but once again the observed concentration at day 7 (the last sample collected) was approaching the limit of detection for the assay. The albumin adduct assay was able to detect the HD-alkylated

tripeptide through day 7 for Patient 2 at concentrations that were approximately four times the limit of quantitation of the assay. The plasma protein adduct assay that was employed at the time of this incident was unable to detect the HD adducts in Patient 2 blood samples, but recent modifications to improve the sensitivity of the method should make it a viable assay for low-level HD exposure.

Each of the methods utilized in this report has certain advantages and disadvantages. The GC–MS is substantially less expensive and requires less operator expertise than the LC–ESI-MS–MS. The GC–MS is a compact, benchtop instrument that is suitable for placement in field forward positions as part of the chemical analysis capabilities of mobile military or homeland security analytical laboratories. The LC–ESI-MS–MS offers greater sensitivity and selectivity. One of the primary limitations of both methods is the need to use the actual chemical warfare agent HD for the in vitro exposure of blood or plasma in order to generate standards. The albumin adduct method has the additional need for an isotopically labeled form of HD to produce the internal standard. The plasma protein assay utilized an isotopically labeled form of TDG as the internal standard out of necessity because the U.S. Army laboratory did not possess labeled HD. Both deuterated and ^{13}C-labeled forms of TDG can be obtained from a commercial source (Cerilliant, Round Rock, TX). Both methods require fairly extensive sample preparation prior to analysis, although the albumin adduct method can process blood samples within several hours. The plasma protein assay incorporates an overnight drying period following the protein precipitation and washing steps. Once the protein has dried, it can be processed for analysis within several hours.

Conclusions

This most recent accidental human exposure to sulfur mustard has provided a rare opportunity to examine blood samples for HD biomarkers and add to the very limited database of knowledge that currently exists regarding this type of chemical exposure. Blood provides several options for assessing potential exposure to sulfur mustard since a variety of different metabolites have been verified in human exposure cases. Two different blood biomarkers of HD exposure from two different patients were successfully examined in this study. Patient 1 suffered extensive chemical burns, and both blood assays were able to detect their respective HD biomarkers in all samples collected over a 42-day period following the exposure. This extends the longest known time period for a positive test for an HD biomarker following a human exposure from the previous period of 22–26 days (16). Patient 2 in this report developed only a single, small blister following the exposure to HD. A low-dose human exposure such as this presents a difficult clinical and analytical challenge to diagnose and verify. The albumin HD adduct assay was able to successfully detect HD biomarkers in all samples collected. These data also provide valuable information linking the measurements obtained using the two analytical methods, which involve systems of relative concentration units based on

standards produced by in vitro spiking.

The results presented here demonstrate the importance of blood sample collection for verification purposes in cases of suspected exposure to sulfur mustard. During the first several days after an exposure, both urine and blood samples should be valuable biomedical samples for verification of exposure. As the time period between an exposure to HD and the collection of a biomedical sample increases, blood specimens will take on greater importance.

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